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## Research Article

# Assessment of Toxicity of Myristicin and 1'-Hydroxymyristicin in HepG2 Cell Line

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## Abstract

**Background and Objective:** Myristicin belongs to a class of potentially toxic chemicals (alkoxy substituted allylbenzenes) and despite the structural analogy with safrole, data on this compound are very controversial and unclear. In this study assessed the cytotoxic and genotoxic potential of myristicin and 1'-hydroxy-myristicin after 24 h of exposure in HepG2 cells. **Methodology:** The compounds were tested up to 600  $\mu$ M concentration, for 24 h. The genotoxicity was assessed with alkaline and neutral comet assay and micronucleus assay. The data were analysed by one-way ANOVA. **Results:** It is to be emphasized that only the synthetic Phase 1 metabolite (1'-hydroxymyristicin) showed a genotoxic effect starting from the concentration of 150  $\mu$ M both in comet and micronucleus tests. However, it is important to point out that the same concentration cause a statistically significant ( $p < 0.001$ ) apoptotic process. **Conclusion:** The consumption of a traditional diet determines very low levels of exposure to the parent myristicin. This fact implies as the primary metabolic pathway the O-demethylation (5-allyl-2,3-dihydroxyanisole) and not to Phase I metabolism, which leads to the conclusion that this substance could not present a significant risk to humans.

**Key words:** Alkenylbenzenes, myristicin, genotoxicity, comet assay, micronucleus, *in vitro* toxicity, apoptosis

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**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Myristicin is an alkoxy-substituted allylbenzene (alkenylbenzene) present in a variety of botanical species such as fennel, parsley, carrot, parsnip, basil, anise, dill, celery and in some spices consumed by humans, such as nutmeg, macis, cinnamon and clove. Myristicin is also found in some food additive oils or in traditional medicine<sup>1,2</sup>.

In the last year's, the consumption of botanical and botanical ingredients has increased, due to the fact that they are used as plant food supplements with the aim of enhancing health. Usually, plant food supplements and phytochemicals are considered safe because of their natural origins<sup>3</sup>. This assumption is not correct because it is known that some herbal preparations can contain individual potential harmful chemicals, among which are alkylbenzenes, in particular, allylalkoxybenzenes, with toxic and well known genotoxic properties<sup>4-6</sup>. Special attention has been given to estragole, methyl eugenole and safrole since, although, at very high levels of exposure, they were found to be genotoxic and carcinogenic in animals<sup>7,8</sup>. The genotoxic potential of myristicin is still questioned even if there are overall evidence that it produces DNA adducts albeit in smaller quantities and less persistent than estragole, safrole and methyleugenol<sup>9,10</sup>. The compound shows neither mutagenic activity in *Salmonella typhimurium* TA100 and TA98 at up to cytotoxic doses with and without metabolic activation nor UDS (Unscheduled DNA Synthesis) in hepatocytes from male fisher 344<sup>11,12</sup>. The discrepancy emerged from *in vivo* and *in vitro* experiments, could likely be a different ability of DNA damage repair activity in the models considered. Myristicin induces apoptotic death in human neuroblastoma SK-N-SH cells accompanied by an accumulation of cytochrome c and by activation of caspase 3<sup>13</sup>. More evidence of apoptosis induction were observed in a study in hamster ovary CHO cells<sup>2</sup> and, more recently, a study revealed that myristicin induces apoptosis in human leukemia K562 cells<sup>14</sup>, besides changes in the mitochondrial membrane potential, the release of cytochrome c, activation of caspase 3 and cleavage of PARP and DNA fragmentation. Furthermore, the same study showed that myristicin down-regulated genes involved in DNA damage response pathways, such as genes for the nucleotide excision repair, the double strand break repair, the DNA damage signaling and stress response<sup>14</sup>.

Alkenylbenzenes can undergo different metabolic pathways (Fig. 1). It is reported in the literature that a notable increase in the formation of the 1'-hydroxy metabolites occurs after an increase in dose of the parent compound, which is accompanied by a shift in metabolic pathways<sup>15,16</sup>. This

metabolite can then become the substrate of sulfotransferase enzymes, going through a reaction of esterification. The esterified metabolite can dissociate and give the reactive carbocation, able to link nitrogenous bases forming adducts with DNA. In human hepatic cells (HepG2), it is evident that myristicin yields DNA adducts quantitatively equivalent to that of safrole<sup>17</sup>. The aim of this work is to study the *in vitro* cyto-genotoxicity of myristicin and 1'-OH myristicin (Fig. 1), using a metabolically active model of human hepatoma cell line (HepG2) and believes that more information is needed to obtain sufficient data for a correct risk assessment.

## MATERIALS AND METHODS

**Chemicals and reagents:** The RPMI-1640 medium, pyruvic acid, L-glutamine, penicillin-streptomycin solution, 3-(4-5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Neutral red solution (0,33%), dimethyl sulfoxide (DMSO), trypsin-EDTA solution 1X, low-melting point agarose (LMA), agarose for routine use, propidium iodide (1mg mL<sup>-1</sup> in water), sodium chloride (NaCl), tris (hydroxymethyl) aminomethane, sodium hydroxide (NaOH), potassium chloride (KCl), Triton X-100, hydrochloric acid (HCl), sodium-citrate, citric acid and sucrose were obtained from Sigma-Aldrich, Italy. Fetal bovine serum (FBS), Sytox green and 6  $\mu$ m fluorescent beads were purchased from Invitrogen Life technologies (Italy).

Myristicin was purchased from Sigma-Aldrich (Milan, Italy) while 1'-hydroxymyristicin was synthesized and provided from Division of Toxicology, Wageningen University (Wageningen, The Netherlands).

Myristicin and 1'-hydroxymyristicin both were dissolved in DMSO and solutions obtained were dissolved 1:1000 in RPMI-1640 medium (Fig. 1).

**Cell cultures:** HepG2 cells, a human hepatocellular carcinoma cell line, were purchased from Istituto zooprofilattico (Brescia, Italy). Cells were maintained in RPMI-1640 medium added with 10% of heat inactivated FBS, 0.01% of pyruvic acid, 0.03% of L-glutamine and 1% penicillin-streptomycin solution and placed at 37°C, under humidified air supplemented with 5% CO<sub>2</sub>.

Confluent monolayers were exposed to myristicin (600  $\mu$ M) or 1'-hydroxymyristicin concentrations (from 50-600  $\mu$ M) in RPMI-1640 medium for 24 h at 37°C.

### Cytotoxicity assessment

**MTT assay:** This assay was conducted according to Schiller *et al.*<sup>18</sup>, HepG2 cells were grown in a 96-well plate,

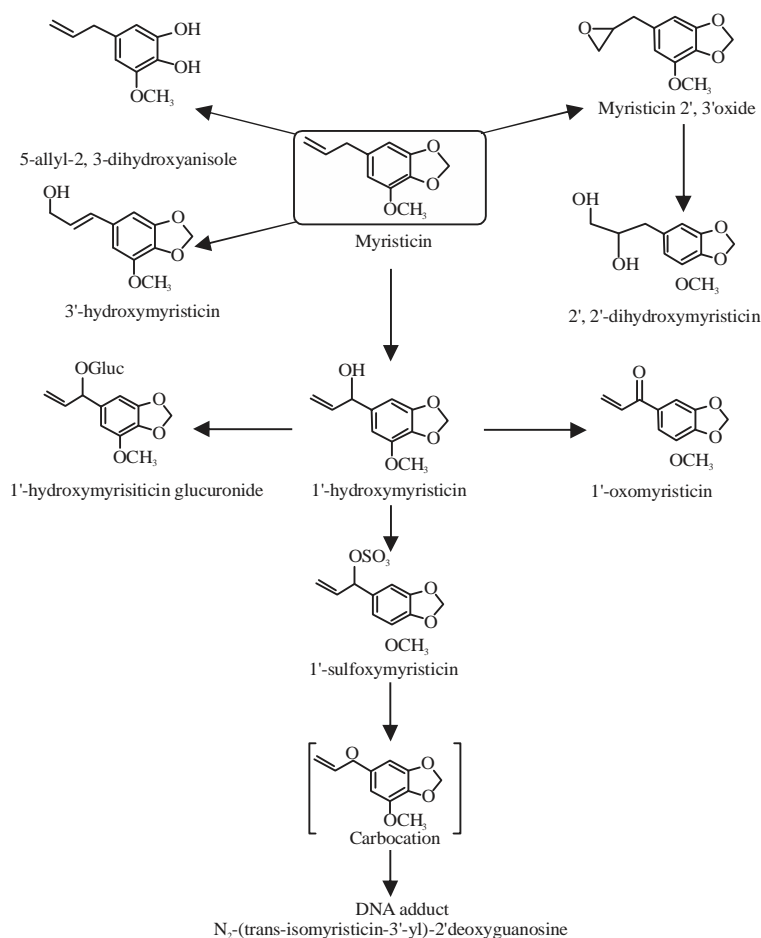


Fig. 1: Proposed metabolic pathways of the alkenylbenzene myristicin<sup>15</sup>

myristicin or 1'-hydroxymyristicin were added and then removed after 24 h. MTT dye (final concentration  $0.5 \text{ mg mL}^{-1}$ ) was added to each well. After removal of MTT solution, cells were lysed with  $150 \text{ }\mu\text{L}$  of DMSO in order to dissolve the formazan crystals. The plate was read at 550 nm with the spectrophotometer (Multilabel counter Victor Wallace 1420, Perkin-Elmer, Italy) and absorbance was determined. Samples with a cell viability less than 50% were subsequently excluded from genotoxicity analysis.

**Neutral red assay:** The neutral Red assay was based on Rodrigues method<sup>19</sup>. HepG2 cells were grown in 96-well plates and subsequently treated. Then plates were washed with PBS and  $200 \text{ }\mu\text{L}$  of the neutral red solution,  $25 \text{ }\mu\text{g mL}^{-1}$  in the culture medium, were added to each well after a centrifugation at 5000 rpm for 5 min. Neutral red (0.33%) was dissolved in culture medium the day before the test and left at  $37^\circ\text{C}$  during the night. The day of the test, plates with neutral red solution was incubated at  $37^\circ\text{C}$  for 3 h and then cells were rinsed with PBS and lysed with a solution containing

citric acid, ethanol and water (1:50:49), in order to let neutral red going out from lysosomes. After 30 min of agitation, plates were read at 550 nm with the spectrophotometer (Multilabel counter Victor Wallace 1420, Perkin-Elmer Italy) and absorbance was determined. The absorbance measured correlates with the number of living cells for each well, considering that each sample is referred to the negative control to which is attributed a 100% cell viability. Samples with a cell viability less than 50% were excluded from genotoxicity analysis.

**Apoptosis evaluation (Annexin V assay):** This assay measures a number of cells that are going toward an apoptotic process, differentiating in early and late stages of this mechanism. Annexin V is a human protein  $\text{Ca}^{2+}$  dependent that for this assay is labeled with a fluorophore. Annexin V has a high affinity for phosphatidylserine (PS), a phospholipid that normally stays on the cytoplasmic surface of cell membrane and that during apoptosis is translocated on the outer side of the membrane, becoming able to be linked by Annexin V. The test was performed with Alexa Fluor 488 Annexin V/Dead cell

apoptosis Kit (Invitrogen). Cells were seeded 24 h before treatment in 60 mm plates at a density of  $6.5 \times 10^5$  cells  $\text{mL}^{-1}$ . After treatment, cells were collected with trypsin and centrifuged for 5 min at 2000 rpm. Cells are then suspended in 1 mL of PBS+5% FBS and counted with trypan blue. A volume of  $10^6$  cells  $\text{mL}^{-1}$  is calculated for each sample and cells are subsequently combined with 100  $\mu\text{L}$  annexin binding buffer 0.5x. Annexin binding buffer 0.5x was obtained with Na citrate (0.1%) Then 5  $\mu\text{L}$  of Annexin V were added to each sample and finally also 1  $\mu\text{L}$  of working solution (propidium iodide dissolved 1:10 in ABB 0.5x) was added to each sample. Samples were left at RT in the dark for 15 min. In the end, 400  $\mu\text{L}$  of ABB 1:10 were added and samples were read in flow cytometry at a wave length of excitation of 496 nm with an emission of 519 nm. Results are expressed as apoptotic cells percentage for each sample.

### Genotoxicity evaluation

**Alkaline comet assay:** Experiments were carried out according to Singh *et al.*<sup>20</sup>, HepG2 cells were plated in 60 mm culture dishes and after 24 h they were exposed to studied compounds. Then the cells were collected with trypsin and centrifuged at 2000 rpm for 5 min. Pellet was suspended in 1 mL of culture medium with a 20 G syringe needle. A total of  $2 \times 10^4$  cells  $\text{mL}^{-1}$  were suspended in 200  $\mu\text{L}$  of 0.5% low melting point agarose (LMA) in PBS and then transferred onto pre coated microscope slides with 1% agarose for routine use in PBS and covered with a cover glass. Slides were stored at 4°C for 10 min, then cover glass was removed and the second layer of LMA was added to each slide. After 10 min at 4°C, slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO, 1% Triton X-100, pH 10) at 4°C for 1 h. Slides were then rinsed with neutralization solution (0.4 M Tris, pH 7.5) and placed in a horizontal gel electrophoresis tank (PBI) filled with ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM Na-EDTA, pH>13) and left this way for 35 min, in order to let DNA unwinding. Then electrophoresis run was done at 300 mA for 45 min, followed by 5 min of neutralization with neutralization solution and finally, slides were fixed with ethanol at -20°C for 5 min. Slides were left to dry at room temperature and then nuclei were stained with propidium iodide (20  $\mu\text{g mL}^{-1}$  in water) and analyzed using fluorescence microscope (Axioplan 2, Zeiss, Milan, Italy) at 25-fold magnification. For each sample, at least 100 randomly selected nucleoids were examined. Images of nucleoids were analyzed with TriTek Comet Score Imaging software 1.5 and tail length, tail moment and % of DNA in the tail were measured. Moreover, nucleoids were classified into 5 different categories according to area, shape

and intensity of fluorescence of their tail. (A: normal nucleoid, B, C, D: damaged nucleoids, E: ghosts).

**Neutral comet assay (NRA):** Slides with a layer of lysed cells and LMA were placed in the horizontal electrophoresis tank with a buffer (pH 8.3) containing 90 mM Tris, 2 mM EDTA, 90 mM boric acid and left this way for 15 min before starting the electrophoretic run at 80 mA for 25 min. Nucleoids were stained with propidium iodide (20  $\mu\text{g mL}^{-1}$  in water) and analyzed using fluorescence microscope (Axioplan 2) at 25-fold magnification<sup>20</sup>.

For each sample, at least 100 randomly selected nucleoids were examined. Images of nucleoids were analyzed with TriTek Comet Score Imaging software 1.5 and tail length, tail moment and % of DNA in the tail were measured.

**Micronucleus assay:** Experiments were done according to Bryce *et al.*<sup>21</sup>, making an analysis of micronuclei in flow cytometry, associated also with a measure of cell viability through fluorescent microspheres (beads). Cell viability measure made through fluorescent beads is considered more accurate than measure obtained with normally used cytotoxicity assays, which can overestimate a number of living cells. The day before treatment, cells were seeded at a density of  $6.5 \times 10^5$  cells  $\text{mL}^{-1}$ . After treatment, a period of 24 h followed in which cells were left in the medium at 37°C, in order to give time to have cell division. The day of the experiment, cells were collected and centrifuged for 5 min at 2000 rpm, then each sample was suspended in 1 mL of PBS+2% FBS and counted by trypan blue method, in order to obtain a quantity of  $5 \times 10^5$  cells  $\text{mL}^{-1}$  for each sample. Calculated volume was suspended in PBS+2% FBS in order to reach a total volume of 1 mL for each sample. After 5 min of centrifugation, 300  $\mu\text{L}$  of propidium iodide (2  $\mu\text{g mL}^{-1}$ ) were added to each tube and samples were left in the dark at real temperature for 10 min. Samples were centrifuged and pellets were suspended in 1 mL of PBS+2% FBS, after another centrifugation of 2000 rpm for 5 min, pellets were left in the dark at RT for 30 min with just 50  $\mu\text{L}$  of supernatant covering them. Then 500  $\mu\text{L}$  of Lysis 1 solution (0.584 mg  $\text{mL}^{-1}$  NaCl, 1.13 mg  $\text{mL}^{-1}$  Na-citrate, 0.3  $\mu\text{L mL}^{-1}$  IGEPAL®CA630, 0.5 mg  $\text{mL}^{-1}$  RNase, 0.4  $\mu\text{M}$  Sytox Green) were added to each sample. After 1 h at RT in the dark, 500  $\mu\text{L}$  of Lysis 2 solution (85.6 g  $\text{mL}^{-1}$  sucrose, 16.4 mg  $\text{mL}^{-1}$  citric acid, 0.4  $\mu\text{M}$  Sytox Green, 2 drops  $\text{mL}^{-1}$  beads) were added to each sample. After at least 30 min in the dark at RT, samples were transferred to FACS tubes and stored at 4°C until flow cytometry analysis. MN number was determined through the acquisition of at least 20,000 gated nuclei for each sample and it is expressed

as fold increase respect negative control. Fold increase  $\geq 3$  was considered a positive result for this test. Nuclei/beads ratio was determined for each sample and referred to that of negative control, in order to have an evaluation of relative cell survival.

**Statistical analysis:** Triplicate experiments were performed with independent samples. The results were analyzed using ANOVA t-test to assess statistical significance, one-way or two-way ANOVA analysis followed by *post-hoc* Dunnett results were considered statistically significant at  $p < 0.05$ . Analysis was carried out using the software package GraphPad Prism version 6.0 (GraphPad Prism Software Inc., La Jolla USA). Statistical differences were considered at the  $p < 0.05$ ,  $p < 0.01$  or  $p < 0.001$  level vs. the control group as indicated in the figures and captions. In the following, the results are expressed as means  $\pm$  standard deviation.

## RESULTS

**MTT and NRA:** Concentrations of myristicin and 1'-hydroxymyristicin suitable to conduct reliable genotoxicity studies were established on the basis of concentrations that did not cause a reduction of more than 50% cell viability. Cells exposed to myristicin (range 50-600  $\mu\text{M}$ ) for 24 h did not show a significant cell viability reduction, both with MTT and NRA up to 600  $\mu\text{M}$  (data not shown). Differently, cells exposed to 1'-hydroxymyristicin, at the same range of concentrations, showed a dramatic viability reduction ( $p < 0.001$ ) starting from 150  $\mu\text{M}$  in MTT test and from 50  $\mu\text{M}$  concentration in NRA (Fig. 2). The MTT and NRA dose-response were very similar.

### Alkaline comet assay (pH>13)

**Myristicin:** Cells were exposed for 24 h to 450 and 600  $\mu\text{M}$  myristicin concentration (Fig. 3). None of the parameters showed a significant difference in respect to control.

**1'-Hydroxymyristicin:** Cells were exposed for 24 h to 50-450  $\mu\text{M}$  concentrations of 1'-hydroxymyristicin (Fig. 4a-c). Tail moment and nucleoids classification showed a significant  $p < 0.05$  difference between cells exposed to 1'-hydroxymyristicin 450  $\mu\text{M}$  and non-treated cells. A significant  $p < 0.05$  increase was measured in the percentage of nucleoids category (A, B, C and D damaged) from 150  $\mu\text{M}$  and above (Fig. 4a-c).

**Neutral comet assay (pH 8):** Using the neutral version of comet assay that identifies double strand damage, a

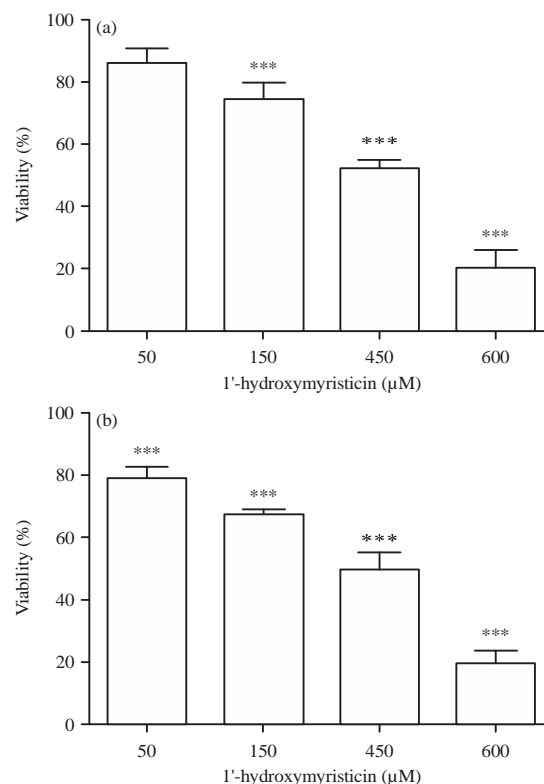


Fig. 2(a-b): Cytotoxicity evaluation, through MTT and Neutral red assay in HepG2 cells exposed to 1'-hydroxymyristicin for 24 h

Data are elaborated through one-way ANOVA (Dunnett's *post hoc* test) analysis. Means  $\pm$  Standard deviation, \*\*\* $p < 0.001$  vs. vehicle, DMSO 0.1%

significant  $p < 0.05$  dose-response increase of DNA damage has been observed only in cells exposed to 1'-hydroxymyristicin, 150 and 450  $\mu\text{M}$  (Fig. 5a, b). In this test, the parameters normally utilized are tail length and tail moment. These results supported the increase of nucleoids E (see alkaline comet assay results) and the reduction of viability already highlighted.

**Micronucleus assay:** The increase of micronucleus frequency, detecting the presence of damaged chromosomes in cells after division, confirms the extent of DNA damage observed in the comet assay (alkaline and neutral test). As shown in Fig. 6, cells exposed only to 150 and 450  $\mu\text{M}$  1'-hydroxymyristicin showed a marked increase in the number of micronuclei largely exceeding the threshold level ( $n = 3$ ) for this test. The decrease of the effect at 450  $\mu\text{M}$  1'-hydroxymyristicin likely due to the cytotoxicity (Fig. 2).

No genotoxic response was elicited by myristicin (600  $\mu\text{M}$ ).

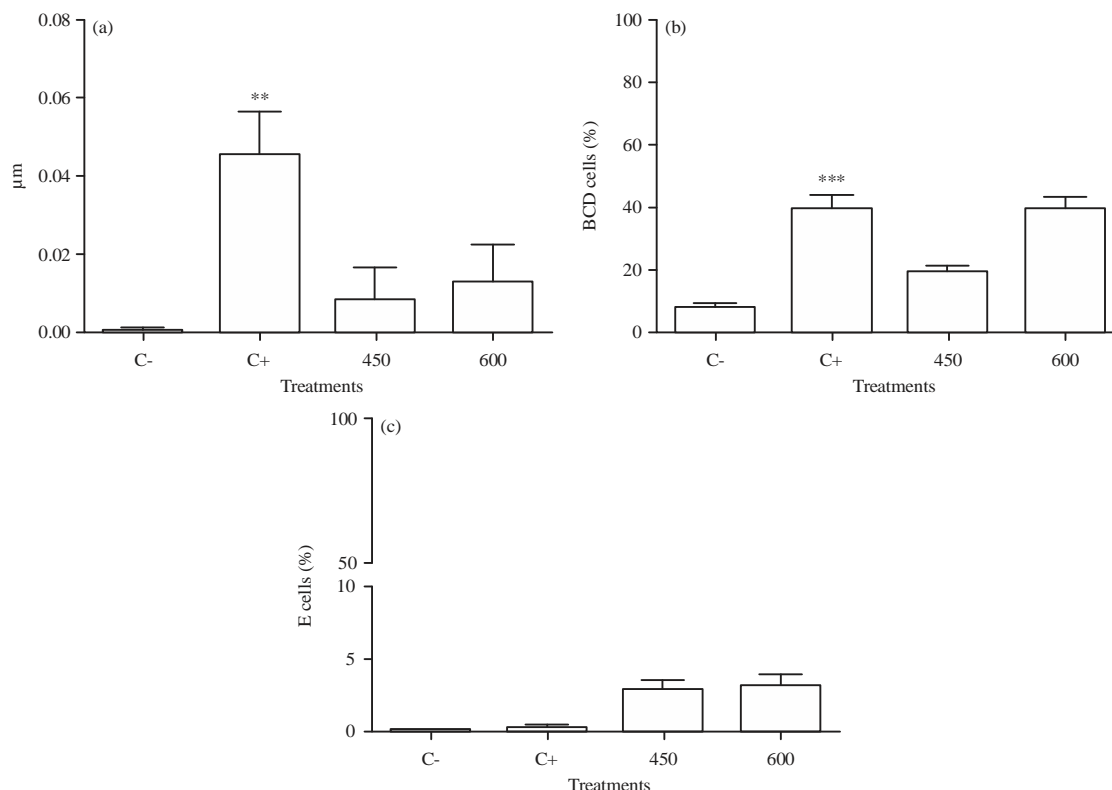


Fig. 3(a-c): Evaluation of genotoxic damage by alkaline comet test (pH>13) in HepG2 cells exposed to myristicin (450-600 μM) for 24 h

Data are elaborated through one-way ANOVA (Dunnett's *post hoc* test) analysis, (a) Tail moment (μm), (b) % BCD nucleoids and (c) % E nucleoids Means ± Standard deviation, \*\*p<0.01, \*\*\*p<0.001 vs C-, vehicle (DMSO 0.1%), C+ positive control (mitomycin 0.1 μg mL<sup>-1</sup>)

**Annexin v assay:** The cytotoxicity and the type of DNA damage have led us to investigate a possible apoptotic effect associated with 1'-hydroxymyristicin treatment.

A significant increase (p<0.01 and p<0.001) in apoptotic cell numbers (both in early Fig. 7a and late apoptotic stage Fig. 7b and therefore not only as phosphatidylserine (PS) expression on the outer leaflet membrane but also as a triggered apoptotic process), was actually observed in cells exposed to concentration of 150 and 450 μM 1'-hydroxymyristicin (Fig. 7a and b). These evidence support the results previously obtained with alkaline comet assay (Nucleoids E) and also with MTT and NRA.

## DISCUSSION

Toxicity of allylbenzenes, constituents of a variety of botanical based food, is strongly dependent on the presence of functional groups that may influence the chemical reactivity accordingly, the biological activity of these natural constituents. The allylbenzene family is very diversified by the presence or absence of alkylation products of their

para-hydroxyl substituents, and/or position of the double bond in the alkyl side chain. Besides this, also minor structural variations may elicit differences in bioactivation/detoxification pathways that can affect the toxicological assessment. This becomes relevant when considering the formation of reactive metabolites. In the scientific literature, there are conflicting data on the toxicity of allylalkoxybenzenes (myristicin, estragole, methyl eugenol and safrole), propenyl alkylbenzenes (anethole, isoeugenol methyl ether), allylhydroxybenzenes (chavicol and eugenol) and propenyl hydroxybenzenes (isochavicol and isoeugenol)<sup>7</sup>.

While estragole, methyl eugenol, safrole and anethole have proved to be hepatotoxic, genotoxic and carcinogenic, the genotoxic and possibly carcinogenic potential of myristicin at equivalent doses is not to be expected. Dose-dependent formation of protein and DNA adducts in liver<sup>22-29</sup> was observed with allyl alkoxy benzenes. Although DNA adducts in the liver of CD1 female mice were isolated, the binding of myristicin to mouse-liver DNA was weaker than those of other compounds such as safrole, estragole and methyleugenol<sup>10</sup>.

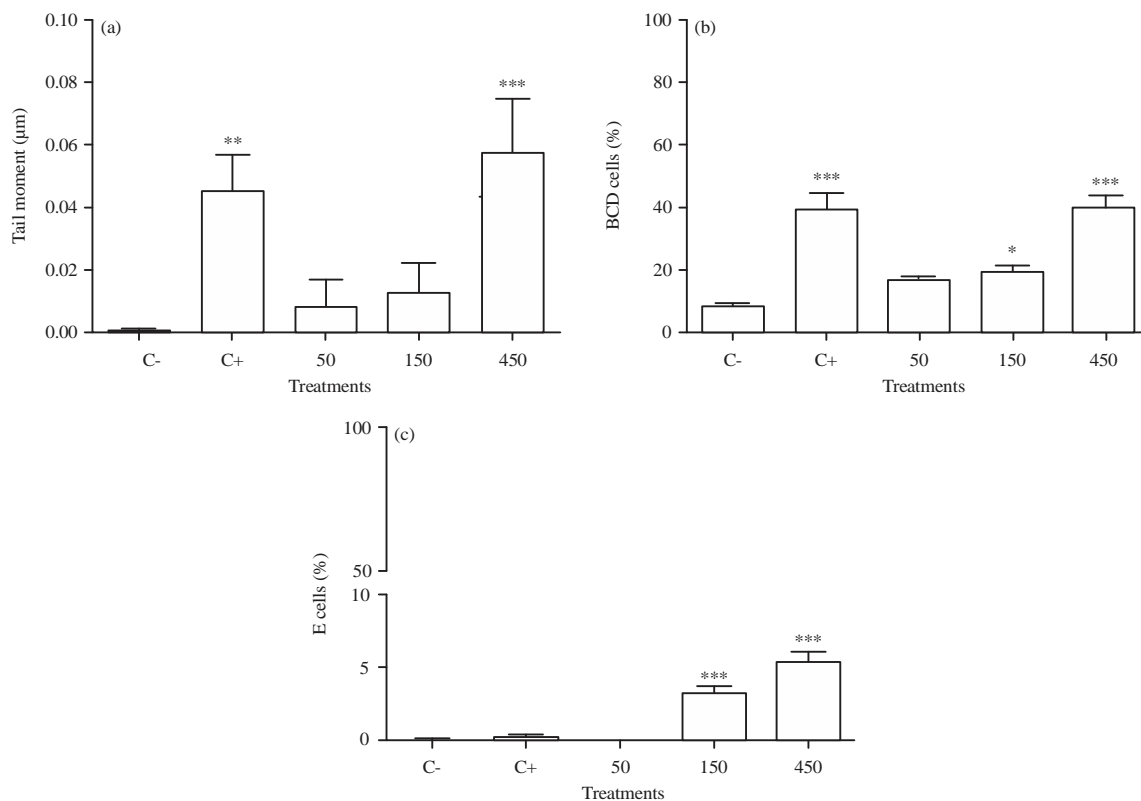


Fig. 4 (a-c): Evaluation of genotoxic damage by alkaline comet test (pH>13) in HepG2 cells exposed to 1'-hydroxymyristicin (50-450 μM) for 24 h

Data are evaluated through one-way ANOVA (Dunnett's *post hoc* test) analysis, (a) Tail moment (μm), (b) % BCD nucleoids and (c) % E nucleoids Means ± Standard deviation, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs C-, vehicle (DMSO 0.1%), C+ positive control (mitomycin 0.1 μg mL<sup>-1</sup>)

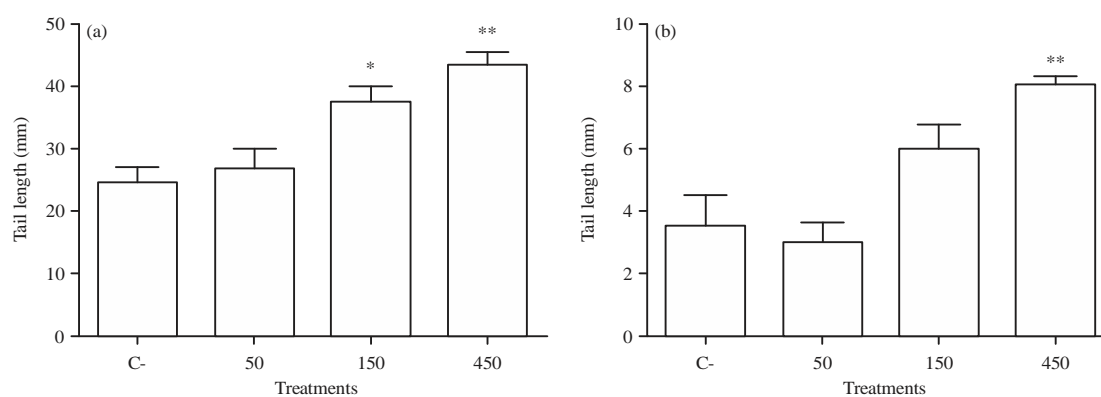


Fig. 5(a-b): Neutral Comet test (pH 8) evaluation in HepG2 cells exposed to 1'-hydroxymyristicin (50-450 μM) for 24 h

Data are evaluated through one-way Anova (Dunnett's *post hoc* test) analysis, (a) Tail length (μm) (b) Tail moment (μm), Means ± Standard deviation, \*p<0.05, \*\*p<0.01 vs C-, vehicle (DMSO 0.1%)

This study tries to clarify the genotoxic potential of myristicin, one of the constituents of nutmeg powder, to which diverse populations are exposed through food and beverages. The human hepatoma line (Hep G2) has retained

the activities of various Phase I And Phase II enzymes which play a crucial role in the activation/detoxication of genotoxic procarcinogens<sup>30</sup>. No carcinogenicity studies of myristicin in animals were available in the literature. Miller *et al.*<sup>25,26</sup>,



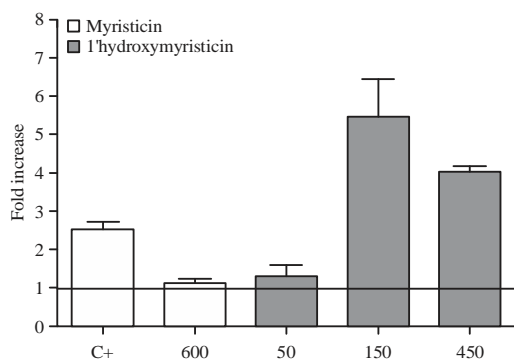


Fig. 6: Evaluation of genotoxic damage through micronucleus test in HepG2 cells exposed to myristicin and 1'-hydroxymyristicin

Detection of micronuclei is made in flow cytometry, C+positive control (mitomycin 0.1  $\mu\text{g mL}^{-1}$ ). Micronuclei values are expressed as fold increase respect negative control (value of 1 on Y axis). A fold increase  $>3$  gives an indication of positive results. Means  $\pm$  Standard deviation

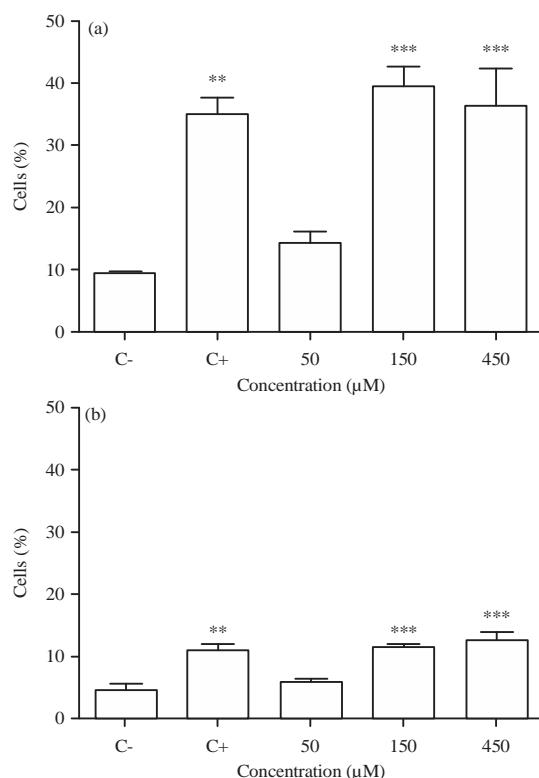


Fig. 7(a-b): Evaluation of apoptotic cells by Annexin V test in HepG2 cells exposed to 1'-hydroxymyristicin for 24 h

Data are elaborated through one-way Anova (Dunnett's *post hoc* test) analysis, (a) Percentage of early apoptotic cells and (b) Percentage of late apoptotic events, Means  $\pm$  Standard deviation, \*\*\*p < 0.001, \*\*p < 0.01, vs C-, vehicle (DMSO 0.1%). C+, positive control (staurosporine 8.56  $\mu\text{M}$ )

performed comprehensive sets of bioassays to characterize the hepatocarcinogenic potential of naturally occurring and synthetic alkylbenzene derivatives including myristicin and its metabolites, intraperitoneally treatment of male B6C3F1 mice 24 h after birth and at days 8, 15 and 22 for a total dose of 4.75  $\mu\text{mol}/\text{mouse}$  did not show carcinogenic effects at 13 months.

The genotoxic potential of alkoxy-substituted allylbenzenes is likely due to the CYP-catalysed formation of the 1'-hydroxy metabolite and subsequent activity of sulfotransferase 1A1 (SULT1A1), that catalyses the formation of the 1'-sulfoxy conjugate. Myristicin *in vivo*, in the range of human food intake, may metabolically be converted mainly by CYP1A1 and 2A6 to epoxy- or hydroxy-derivatives that undergo glucuronidation and are readily excreted. At high doses in rodents, O-demethylation becomes saturated and then takes place 1'-hydroxylation and epoxidation of the allyl side-chain. This change in the balance of metabolic pathways, at high doses, leads to a predominant formation of 1'-hydroxy-metabolites and the subsequent formation of 1'-sulfoxy metabolites by SULT 1A1 and SULT 1C2 that have been associated with the genotoxicity and carcinogenicity<sup>7,31</sup>.

The unstable sulfate ester forms a reactive electrophilic intermediate (carbonium ion or quinolinium cation), which binds to proteins and DNA. Sulfate inhibition studies and *in vivo-in vitro* unscheduled DNA synthesis (UDS) assays of myristicin, elemicin, estragole, methyl eugenol and the 1'-hydroxy metabolites of estragole and methyl eugenol<sup>12,21,32</sup> provide additional evidence that the sulfate ester of the 1'-hydroxy metabolite is the ultimate toxic metabolite in animal. Data related to the safety of estragole and safrole, structurally related to myristicin, indicate that at low dose levels (below 1-10 mg kg b.wt., in rodents and humans), allylalkoxybenzenes are rapidly cleared from the body, with O-demethylation being the major metabolic route. Metabolic shifting from O-demethylation to 1'-hydroxylation results in increased formation of 1'-hydroxymetabolite and accordingly of the toxic reactive electrophilic 1'-sulfoxy conjugate metabolite at higher dose level (30-300 mg kg b.wt.,)<sup>31</sup>.

Our data showed no genotoxic potential of myristicin in comparison to other alkenylbenzenes with similar structures (safrole, estragole)<sup>33,34</sup>. Besides, myristicin does not elicit any cytotoxicity differently from its 1'-hydroxymetabolite that elicited cytotoxic, genotoxic and apoptotic effects from a concentration of 150  $\mu\text{M}$ . The appearance of the apoptotic effect must be considered because it can mask the genotoxic effect. It is also evident that 600  $\mu\text{M}$  myristicin in our experimental conditions does not generate a sufficient 1'-hydroxymyristicin quantity to give the final toxicant product.

## CONCLUSION

Our data confirms that there was no genotoxic potential of myristicin. The fact that only hydroxyl metabolite is genotoxic in a narrow range of doses, underlines that the mechanism of carbocation formation (1'-sulfoxy conjugate toxic metabolite production) is necessary for genotoxic activity.

This is an important point to consider in the risk assessment of dietary exposure to myristicin, which is mainly carried through consumption of the spices nutmeg and mace and of non alcoholic beverages. The average exposure for myristicin may be as high as to 162 µg/day (3/684 µg/day lower and upper limits equal to 0.05 and 11.4 µg kg<sup>-1</sup> b.wt./day, respectively for an adult of the average weight of 60 kg) in Europe. The results lead to the conclusion that myristicin presents no significant risk to humans through consumption of a traditional diet because the very low levels of exposure. The low doses cause essentially the primary involvement of the O-demethylation leading to a safer metabolic path.

## SIGNIFICANCE STATEMENTS

This study shows that data concerning to alkenylbenzene compound are limited and inconsistent. This study provided additional information regarding the myristicin genotoxicity and its metabolism.

## REFERENCES

1. Hallstrom, H. and A. Thuvander, 1997. Toxicological evaluation of myristicin. *Nat. Toxins*, 5: 186-192.
2. Martins, C., C. Doran, A. Lares, J. Rueff and A.S. Rodrigues, 2011. Genotoxic and apoptotic activities of the food flavourings myristicin and eugenol in AA8 and XRCC1 deficient EM9 cells. *Food Chem. Toxicol.*, 49: 385-392.
3. Van den Berg, S.J.P.L., A. Punt, A.E.M.F. Soffers, J. Vervoort, S. Ngeleja, B. Spenkelink and I.M.C.M. Rietjens, 2012. Physiologically based kinetic models for the alkenylbenzene elemicin in rat and human and possible implications for risk assessment. *Chem. Res. Toxicol.*, 25: 2352-2367.
4. SCF., 2001. Opinion of the scientific committee on food on the safety of the presence of saffrole (1-allyl-3,4-methylene dioxy benzene) in flavourings and other food ingredients with flavouring properties. Scientific Committee on Food, SCF/CS/FLAV/FLAVOUR/6 ADD3 Final, pp: 1-10.
5. SCF., 2001. Opinion of the scientific committee on food on estragole (1-Allyl-4-Methoxybenzene). Scientific Committee on Food, SCF/CS/FLAV/FLAVOUR/6 ADD2 Final, September 26 2001, pp: 1-10.
6. SCF., 2001. Opinion of the scientific committee on food on methyleugenol (4-Allyl-1,2-Dimethoxybenzene), Scientific Committee on Food, SCF/CS/FLAV/FLAVOUR/4 ADD1 Final, September 26, 2001, pp: 1-10.
7. Rietjens, I.M.C.M., S.M. Cohen, S. Fukushima, N.J. Gooderham and S. Hecht *et al.*, 2014. Impact of structural and metabolic variations on the toxicity and carcinogenicity of hydroxy- and alkoxy-substituted allyl- and propenylbenzenes. *Chem. Res. Toxicol.*, 27: 1092-1103.
8. Van den Berg, S.J.P.L., P. Restani, M.G. Boersma, L. Delmulle and I.M.C.M. Rietjens, 2011. Levels of genotoxic and carcinogenic compounds in plant food supplements and associated risk assessment. *Food Nutr. Sci.*, 2: 989-1010.
9. Phillips, D.H., M.V. Reddy and K. Randerath, 1984. 32P-post-labelling analysis of DNA adducts formed in the livers of animals treated with saffrole, estragole and other naturally-occurring alkenylbenzenes. II. Newborn male B6C3F1 mice. *Carcinogenesis*, 5: 1623-1628.
10. Randerath, K., R.E. Haglund, D.H. Phillips and M.V. Reddy, 1984. 32P-post-labelling analysis of DNA adducts formed in the livers of animals treated with saffrole, estragole and other naturally-occurring alkenylbenzenes. I. adult female CD-1 mice. *Carcinogenesis*, 5: 1613-1622.
11. Marcus, C. and E.P. Lichtenstein, 1982. Interactions of naturally occurring food plant components with insecticides and pentobarbital in rats and mice. *J. Agric. Food Chem.*, 30: 563-568.
12. Hasheminejad, G. and J. Caldwell, 1994. Genotoxicity of the alkenylbenzenes alpha- and beta-asarone, myristicin and elemicin as determined by the UDS assay in cultured rat hepatocytes. *Food Chem. Toxicol.*, 32: 223-231.
13. Lee, B.K., J.H. Kim, J.W. Jung, J.W. Choi and E.S. Han *et al.*, 2005. Myristicin-induced neurotoxicity in human neuroblastoma SK-N-SH cells. *Toxicol. Lett.*, 157: 49-56.
14. Martins, C., C. Doran, I.C. Silva, C. Miranda, J. Rueff and A.S. Rodrigues, 2014. Myristicin from nutmeg induces apoptosis via the mitochondrial pathway and down regulates genes of the DNA damage response pathways in human leukaemia K562 cells. *Chem.-Biol. Interact.*, 218: 1-9.
15. Al-Malahmeh A.J., A. Al-Ajlouni, S. Wesseling, A.E.M.F. Soffers and A. Al-Subeih *et al.*, 2017. Physiologically based kinetic modeling of the bioactivation of myristicin. *Arch. Toxicol.*, 91: 713-734.
16. Zangouras, A., J. Caldwell, A.J. Huttand and R.L. Smith, 1981. Dose dependent conversion of estragole in the rat and mouse to the carcinogenic metabolite, 1'-hydroxyestragole. *Biochem. Pharmacol.*, 30: 1383-1386.

17. Zhou, G.D., B. Moorthy, J. Bi, K.C. Donnelly and K. Randerath, 2007. DNA adducts from alkoxyallylbenzene herb and spice constituents in cultured human (HepG2) cells. *Environ. Mol. Mutagen.*, 48: 715-721.
18. Schiller, C.D., A. Kain, K. Mynett and A. Gescher, 1992. Assessment of viability of hepatocytes in suspension using the MTT assay. *Toxicol. In Vitro*, 6: 575-578.
19. Rodrigues, R.M., M. Bouhifd, G. Bories, M.G. Sacco and L. Gribaldo *et al.*, 2013. Assessment of an automated *in vitro* basal cytotoxicity test system based on metabolically-competent cells. *Toxicol. In Vitro*, 27: 760-767.
20. Singh, N.P., M.T. McCoy, R.R. Tice and E.L. Schneider, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell. Res.*, 175: 184-191.
21. Bryce, S.M., J.C. Bemis, S.L. Avlasevich and S.D. Dertinger, 2007. *In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity. *Mutat. Res./Genet. Toxicol. Environ. Mutagen.*, 630: 78-91.
22. Boberg, E.W., E.C. Miller, J.A. Miller, A. Poland and A. Liem, 1983. Strong evidence from studies with brachymorphic mice and pentachlorophenol that 1'-sulfooxy safrole is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole in mouse liver. *Cancer Res.*, 43: 5163-5173.
23. Drinkwater, N.R., E.C. Miller, J.A. Miller and H.C. Pitot, 1976. Hepatocarcinogenicity of estragole (1-allyl-4-methoxybenzene) and 1'-hydroxyestragole in the mouse and mutagenicity of 1'-acetoxyestragole in bacteria. *J. Natl. Cancer Inst.*, 57: 1323-1331.
24. Swanson, A.B., E.C. Miller and J.A. Miller, 1981. The side-chain epoxidation and hydroxylation of the hepatocarcinogens safrole and estragole and some related compounds by rat and mouse liver microsomes. *Biochim. Biophys. Acta*, 673: 504-516.
25. Miller, J.A., E.C. Miller and D.H. Phillips, 1982. The Metabolic Activation and Carcinogenicity of Alkenylbenzenes that Occur Naturally in Many Spices. In: *Carcinogens and Mutagens in the Environment*, Stich, H.F. (Ed.), CRC Press, USA, pp: 83-96.
26. Miller, E.C., A.B. Swanson, D.H. Phillips, L. Fletcher, A. Liem and J.A. Miller, 1983. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. *Cancer Res.*, 43: 1124-1134.
27. Gardner, I., P. Bergin and P. Stening, 1995. Protein adducts derived from methyleugenol. Proceedings of the Meeting Report of the 4th International Ixx Meeting, (MRIIM'95), International Society for the Study of Xenobiotics.
28. Gardner, I., P. Bergin, P. Stening, J.G. Kenna and J. Caldwell, 1996. Immunochemical detection of covalently modified protein adducts in livers of rats treated with methyleugenol. *Chem. Res. Toxicol.*, 9: 713-721.
29. Daimon, H., S. Sawada, S. Asakura and F. Sagami, 1998. *In vivo* genotoxicity and DNA adduct levels in the liver of rats treated with safrole. *Carcinogenesis*, 19: 141-146.
30. Knasmüller, S., W. Parzefall, R. Sanyal, S. Ecker and C. Schwab *et al.*, 1998. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. *Mutat. Res./Fundam. Mol. Mech. Mutagen.*, 402: 185-202.
31. Smith, R.L., T.B. Adams, J. Doull, V.J. Feron and J.I. Goodman *et al.*, 2002. Safety assessment of allylalkoxybenzene derivatives used as flavouring substances-methyl eugenol and estragole. *Food Chem. Toxicol.*, 40: 851-870.
32. Chan, V.S. and J. Caldwell, 1992. Comparative induction of unscheduled DNA synthesis in cultured rat hepatocytes by allylbenzenes and their 1'-hydroxy metabolites. *Food Chem. Toxicol.*, 30: 831-836.
33. Rietjens, I.M.C.M., M.G. Boersma, H. van der Woude, S.M.F. Jeurissen, M.E. Schutte and G.M. Alink, 2005. Flavonoids and alkenylbenzenes: Mechanisms of mutagenic action and carcinogenic risk. *Mutat. Res./Fundam. Mol. Mech. Mutagen.*, 574: 124-138.
34. Rietjens, I.M.C.M., W. Slob, C. Galli and V. Silano, 2008. Risk assessment of botanicals and botanical preparations intended for use in food and food supplements: emerging issues. *Toxicol. Lett.*, 180: 131-136.